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Award Number: W81XWH-08-1-0572

TITLE: RNAi AS A ROUTINE ROUTE TOWARD BREAST CANCER THERAPY

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REPORT DATE: September 2010

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)* 01-09-2010	**2. REPORT TYPE** Annual	**3. DATES COVERED** *(From - To)* 1 Sep 2009-31 AUG 2010

4. TITLE AND SUBTITLE RNAi AS A ROUTINE ROUTE TOWARD BREAST CANCER THERAPY	**5a. CONTRACT NUMBER**
	5b. GRANT NUMBER W81XWH-08-1-0572
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S) GREGORY HANNON E-Mail: hannon@cshl.edu	**5d. PROJECT NUMBER**
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Cold Spring Harbor Laboratory Cold Spring Harbor, NY 11724	**8. PERFORMING ORGANIZATION REPORT NUMBER**
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	**10. SPONSOR/MONITOR'S ACRONYM(S)**
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
No abstract provided.

15. SUBJECT TERMS
RNAi, sequencing

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON** USAMRMC
a. REPORT U	**b. ABSTRACT** U	**c. THIS PAGE** U	UU	7	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction

The goal of this innovator award is to continue to develop and apply RNAi-based screening methods to discover new routes toward breast cancer therapy. The project has three sets of goals. First is to integrate genomic and genetic information on available breast cancer cell lines to identify tumor-specific vulnerabilities and to understand genetic determinants of therapy resistance. Second is to probe the roles of breast cancer stem cells, with a particular emphasis on microRNAs. The third is to examine regions that determine familial susceptibility to breast cancer by applying novel, focal re-sequencing methods developed in the laboratory.

Body

Third and fourth generation RNAi libraries

Although our second-generation shRNA library was a major improvement over the first version, we knew that even given improved designs, only a fraction of constructs showed highly penetrant knockdown. We considered mounting an effort toward large-scale validation, as is being carried out at the Broad Institute, but realized that the time, cost, infrastructure and staff needed were beyond our means. We therefore needed a more clever, higher throughput approach toward measuring shRNA efficiency. This led to the development of the so-called "sensor assay" for shRNA potency, a project we undertook in collaboration with Scott Lowe's lab at CSHL (see Fellmann et al., Mol. Cell, 2010). In essence, we worked with Agilent to enable large-scale synthesis of extremely long oligonucleotides (now up to 300 bases) in situ on glass slide microarrays. For this purpose, we use ~200nt oligos which carry both the shRNA sequence and a region of the target mRNA surrounding its recognition site. This single element is then inserted into a vector and repaired with a second insert to form two independent transcription units, one of which expresses the shRNA from an inducible promoter and one of which expresses the target constitutively. In this way, each individual cell reports the activity of a particular shRNA, with the readout of efficiency being a change in the expression of a fluorescent reporter. Using this method, we were able to measure the activity of more than 20,000 different shRNAs in a single pool.

In the published work, we mainly proved the principle of the method and tiled a few genes of high interest. Since that time, my laboratory has constructed a set of shRNA sensors for all human genes and has nearly completed the analysis of shRNA efficiency for this collection (9/12 libraries have been analyzed). Not only did this yield an average of two validated highly efficient shRNAs per gene but it also demonstrated that there were distinct, sequence-based rules that determine shRNA efficacy. Simon Knott, a new postdoctoral fellow in my laboratory, has nearly completed work on a bioinformatic algorithm, trained on the sensor data, which can predict the outcome of a sensor assay with

90% accuracy. In this way, we can combine the power of both the functional assay and the bioinformatics algorithm in the design of a fourth generation library, where virtually every shRNA will be fully optimized. As always, we will make these tools available to the community.

shRNA screens for new therapeutic targets

Last year, we reported that we had acquired nearly 100 breast cancer cell lines with full annotation (transcriptome, CNVs etc) for the purpose of searching for both genetic dependencies and modifiers of response to appropriate targeted therapies (e.g. anti-estrogen for luminal lines etc). Full genome screens were somewhat delayed since the IC20 and IC80 data that we received from the Slamon and Gray labs were not really transferrable to our screening conditions. Therefore, we had to take a step backward and re-determine these values prior to the entry of each line into our screening pipeline. The status of screens to date are given in Table 1.

Table 1.

cell line	conditions	# of timepoints
JIMT1	straight lethal	T=11
JIMT1	lapatinib IC20	T=11
MDAMB453	straight lethal	T=5
MDAMB453	lapatinib IC20	T=5
MDAMB468	straight lethal	T=4
Hs578T	straight lethal	T=7
MDAMB361	straight lethal	currently at T=2
MCF7 Parental + E2	straight lethal	T=4
MCF7 Parental + E2	without E2	T=4
MCF7 Parental + E2	without E2/ plus tamoxifen	T=3
MCF7-EDR	with E2	T=4
MCF7-EDR	without E2	T=4
MCF7-TAMR	with E2	currently at T=2
MCF7-TAMR	without E2/ plus tamoxifen	currently at T=2
ZR-75-1-TAMR	with E2	currently at T=2
ZR-75-1-TAMR	without E2/ plus tamoxifen	currently at T=2

Though we had industrialized the screening pipeline itself, we had not industrialized DNA preparation and analysis of the screens. While we still need to rebuild our computational pipelines to handle the level of data we are generating, we have standardized DNA preparation and amplification of integrated viruses for analysis by Illumina (as opposed to our prior methods, which used arrays). Therefore, I feel strongly that the coming year will produce quite a lot of data which can then be analyzed in consultation with our various clinical collaborators (e.g. Slamon, Wicha, Osborne etc).

microRNAs in cancer stem cells

We have built upon our discovery of a number of microRNAs that characterize stem cells present within a continuous mouse mammary cell line (commaD) to show that both let-7 and miR-93 impact both normal stem cell self-renewal and the ability of continuous human cancer lines to engraft and form tumors. In collaboration with Max Wicha, we have investigated in particular miR-93 over the past year. We have shown that in basal lines, particularly the claudin low class, expression of miR-93 negates their ability to form tumors upon injection into immunocompromised mice without affecting overall proliferation rates in vitro. Moreover, in established tumors, induction of miR-93 cooperates with chemotherapy to enhance its effects. Identification of miR-93 targets points strongly to well-established stem cell self-renewal pathways, validating our general hypothesis. Of note, miR-93 expression has the opposite impact on luminal tumors, actually enhancing their tumorigenic potential. This result highlights the biological differences between these tumor types and demonstrates that the effect of specific microRNAs and their targets can be context specific.

Studies of normal stem cells and mouse mammary development

Over the past year, we have completed a survey of normal mammary cell types from the expression and transcriptomic perspective and have nearly completed full genome bisulfite sequencing to establish the epigenetic signatures of MaSC, luminal and basal progenitor cells, mature myoepithelial cells, and ductal and alveolar luminal cells. These will serve as useful reference points not only for classifying human tumors but also for classifying mouse models. A focus of our search has been a refined definition of the mammary stem cell. We have taken advantage of a histone-H2B-GFP transgenic mouse, which enables the marking of slow-cycling populations in many tissues. It has been demonstrated that these slow cycling cells represent stem cells in blood, hair follicle, and several other tissues. Isolating H2B positive cells from the mammary gland (following a pulse/chase protocol), we find that these cells have a several-fold enhanced capacity to reform the mammary tree upon transplantation – the most rigorous assay of stem cell potential. These cells also have an expression profile enriched for known stem cell expression signatures.

We searched in our expression profiles for cell surface markers that might characterize mammary stem cells. The underlying notion is that these might help lend further specificity to the marker set that is currently used for MaSC isolation. We also felt that such markers could potentially enable selective targeting of tumor initiating populations, if they similarly marked those cells. Thus far, two markers, CD1 and CD59 have emerged as candidates in both regards. Both enrich cells with repopulating potential, though the CD1 population seems more specific at present. There are also indications from the literature of potential anti-tumor effects of CD1, which we might now be able to explain.

These two markers and their relevance in both normal mouse mammary development and in human cancer will form major foci for the coming year.

Reportable Outcomes

Fellmann, C., J. Zuber, et al. (2011). "Functional identification of optimized RNAi triggers using a massively parallel sensor assay." Molecular Cell **41**(6): 733-746.

Premsrirut, P. K., L. E. Dow, et al. (2011). "A rapid and scalable system for studying gene function in mice using conditional RNA interference." Cell **145**(1): 145-158.

Conclusions

We continue to make progress toward our goals. Though there were some setbacks in the proposed shRNA screens, we have overcome these and are now well on our way to producing very important datasets. We have also made significant strides in our studies of normal stem cells and their tumorigenic counterparts.